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(54) Title: SURFACTANT–LIPASE COMPLEX IMMOBILIZED ON INSOLUBLE MATRIX

(57) Abstract

A lipase or phospholipase preparation which includes an insoluble matrix and a surfactant–coated lipase complex immobilized onto the insoluble matrix. A method of preparing an insoluble matrix–immobilized surfactant–coated lipase complex which includes the steps of (a) contacting a lipase, an insoluble matrix and a surfactant in an aqueous solution; and (b) providing conditions for a formation of the matrix–immobilized surfactant–coated lipase complex.

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SUMMARY OF THE INVENTION

According to the present invention there is provided a surfactant-coated lipase complex immobilized on an inorganic matrix. There are further provided a method of preparing an insoluble matrix-immobilized surfactant-coated lipase complex and a process of modifying oils and fats using same.

According to further features in preferred embodiments of the invention described below, there is provided a lipase preparation comprising an insoluble matrix and a surfactant-coated lipase complex immobilized onto the insoluble matrix.

According to still further features in the described preferred embodiments there is provided a method of preparing an insoluble matrix-immobilized surfactant-coated lipase complex comprising the steps of (a) contacting a lipase, an insoluble matrix and a surfactant in an aqueous solution; and (b) providing conditions for a formation of the matrix-immobilized surfactant-coated lipase complex.

According to still further features in the described preferred embodiments the lipase is first contacted with the insoluble matrix and thereafter with the surfactant.

According to still further features in the described preferred embodiments the lipase is first contacted with the surfactant and thereafter with the insoluble matrix.

According to still further features in the described preferred embodiments the method further comprising the step of (c) separating the matrix-immobilized surfactant-coated lipase complex from the aqueous solution.

According to still further features in the described preferred embodiments the method further comprising the step of (d) drying the matrix-immobilized surfactant-coated lipase complex.

According to still further features in the described preferred embodiments the drying is effected by freeze drying.

According to still further features in the described preferred embodiments following drying the matrix-immobilized surfactant-coated lipase complex, the matrix-immobilized surfactant-coated lipase complex includes less than 100 parts per million water content by weight.

According to still further features in the described preferred embodiments the aqueous solution is a buffered aqueous solution.

According to still further features in the described preferred embodiments contacting of the lipase, insoluble matrix and surfactant in the aqueous solution

According to still further features in the described preferred embodiments contacting the matrix-immobilized surfactant-coated lipase complex with the substrates is effected in presence of an organic solvent.

According to still further features in the described preferred embodiments the matrix-immobilized surfactant-coated lipase complex represents 2-30 weight percent of the substrates.

According to still further features in the described preferred embodiments the oil/fat substrates are selected from the group consisting of liquid oils and solid fats.

According to still further features in the described preferred embodiments the oil is selected from the group consisting of olive oil, fish oil, palm oil, cotton seeds oil, sunflower oil, *Nigella sativa* oil, canola oil and corn oil in their native and hydrogenated forms.

According to still further features in the described preferred embodiments the oils/fats having the changed physical properties are triacylglycerols.

According to still further features in the described preferred embodiments there is provided a triacylglycerol prepared according to the process described herein, the triacylglycerol serves an application selected from the group consisting of cocoa butter substitute, human milk fat-like, triglycerides for special diets and structured triglycerides for medical applications.

According to still further features in the described preferred embodiments there is provided a preparation comprising a lipase and an organic solvent, the lipase possessing both esterification and hydrolysis activities with respect to substrates yielding esterification and hydrolysis products, respectively, the hydrolysis products represent less than about 7 weight percent of the products.

According to still further features in the described preferred embodiments the surfactant-coated lipase complex is immobilized onto the insoluble matrix via a mechanism selected from the group consisting of physical (hydrophobic) interaction, ionic interaction and covalent immobilization.

According to still further features in the described preferred embodiments the insoluble matrix is selected from the group consisting of an inorganic insoluble matrix and an organic insoluble matrix.

According to still further features in the described preferred embodiments the inorganic insoluble matrix is selected from the group consisting of alumina, diatomaceous earth, celite, calcium carbonate, calcium sulfate, ion-exchange resin, silica gel and charcoal.

According to still further features in the described preferred embodiments the organic solvent is selected from the group consisting of *n*-hexane, toluene, iso-octane, *n*-octane, benzene, cyclohexane and di-iso-propylether.

According to still further features in the described preferred embodiments the catalytic activity is selected from the group consisting of esterification, acidolysis, inter-esterification and trans-esterification of oils and fats and alcoholysis of triglycerols and fatty alcohols.

According to still further features in the described preferred embodiments the preparation has 1,3-positional specificity with respect to triacylglycerols.

According to another embodiment of the invention there is provided a process of resolving or synthesizing a chiral product from a racemic or prochiral compound comprising the step of contacting an insoluble matrix-immobilized surfactant-coated lipase complex with the racemic or prochiral compound, thereby obtaining the chiral product.

The present invention successfully addresses the shortcomings of the presently known configurations by providing an insoluble matrix-immobilized surfactant-coated lipase complex, a method of preparing same and a process of modifying oils and fats using the matrix-immobilized surfactant-coated lipase complex.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1a presents an inter-esterification acidolysis reaction catalyzed by lipase with 1,3-positional specificity. P represents glycerol bound palmitic acid, C represents glycerol bound capric acid. PA and CA represent free palmitic and capric acids, respectively.

FIG. 1b presents a trans-esterification reaction catalyzed by lipase with 1,3-positional specificity. P represents glycerol bound palmitic acid and C represents glycerol bound capric acid.

FIG. 2 depicts the chemistry associated with covalent immobilization of lipase to Eupergit C 250L followed by coating the covalently immobilized enzyme with a surfactant.

FIG. 3 presents inter-esterification reaction profiles of physically immobilized lipases. Reaction conditions were 50 mg tripalmitin, 35 mg capric acid and 20 mg surfactant-coated lipase (Saiken 100 - triangles, or lilipase A10-

methods: (i) immobilization through hydrophobic (physical) adsorption on inorganic or organic insoluble matrices; (ii) immobilization through ionic interactions on various ion exchange resins (organic or inorganic matrices); and (iii) immobilization through covalent immobilization to insoluble matrix such as Eupergit (organic matrix).

The immobilized surfactant-coated lipases prepared according to the procedures described herein were used to catalyze inter-esterification reactions between triglycerides and fatty acids, one-step alcoholysis reactions between triglycerides and fatty alcohols for production of wax esters, and also trans-esterification reactions between two different triglyceride molecules or between two different oils.

The results indicate that coating lipases with a lipid surfactant, such as, but not limited to, fatty acid sugar ester types, lead to activation of the lipases for use in organic synthesis and in most cases the modification process converts relatively inactive crude lipases to highly active biocatalysts.

To develop an efficient enzymatic inter/trans-esterification bioreactor from which the lipase enzyme can be easily recovered or used continuously, surfactant-lipase complexes immobilized on organic and inorganic matrices, were used.

It was found that surfactant-coated lipases immobilized on an organic or inorganic matrix showed high inter/trans-esterification activity and only slight activity losses in five consecutive inter-esterification runs using the same biocatalyst batch.

The immobilized surfactant-lipase complexes prepared according to the present invention were used for the preparation of structured triglycerides which have potential applications in medicine. The triglycerides of interest that were synthesized according to the method of the present invention were basically produced by inter-esterifying long-chain triglycerides, such as the hard fraction of palm oil, with short-chain fatty acids such as capric acid. Immobilized surfactant-coated lipase catalyzed reactions yielded predominantly products with 1,3-positional specificity for the triglycerides of interest.

Mono- and di-glycerides were also produced in a hydrolysis side reaction and their percentage was typically less than 7 weight percent of the initial triglycerides concentration.

The operational stability of surfactant-lipase complexes immobilized on different solid matrices was very high and no significant enzyme activity losses were observed.

trilaurate, trimyristate, tripalmitate or tristearate. The hydrophilic moiety is preferably a sugar, such as, but not limited to, sorbitol, sucrose, glucose and lactose, a phosphate group or a carboxylic group. Typically, the fatty acid and the hydrophilic moiety are conjugated via an ester bond.

According to another preferred embodiment of the invention the lipase is derived from a micro or multicellular organism. Species known to be used for lipase extraction include *Burkholderia sp., Candida antarctica B, Candida rogosa, Pseudomonas sp., Candida antractica A,* Porcine, *Humicola sp., Mucor miehei, Rhizopus javan, Pseudomonas fluor, Candida cylindrcae, Aspergillus niger, Rhizopus oryzae, Mucor javanicus, Rhizopus sp., Rhizopus japonicus and Candida antarctica.*

According to a preferred embodiment of the invention the lipase preparation maintains lipase catalytic activity in an organic solvent. Lipase catalytic activity include hydrolysis, esterification, inter-esterification, trans-esterification, acidolysis and alcoholysis, preferably with 1,3-positional specificity with respect to triacylglycerols.

The organic solvent is typically a hydrophobic solvent, such as, but not limited to, *n*-hexane, toluene, iso-octane, *n*-octane, benzene, cyclohexane and di-iso-propylether.

Further according to the present invention there is provided a method of preparing an insoluble matrix-immobilized surfactant-coated lipase complex. The method includes the following method steps, wherein in a first step a lipase, an insoluble matrix and a surfactant are contacted in an aqueous solution, preferably a buffered solution. Second, conditions (e.g., sonication) are provided for the formation of the matrix-immobilized surfactant-coated lipase complex. Two alternative schemes are available in this respect. In the first the lipase is first interacted with the surfactant and only thereafter the surfactant coated lipase is interacted with the matrix. Whereas in the second, the lipase is first interacted with the matrix and only thereafter the matrix immobilized lipase is interacted with the surfactant.

According to a preferred embodiment, the method further includes the step of separating the matrix-immobilized surfactant-coated lipase complex from the aqueous solution.

According to still another preferred embodiment of the invention the method further includes the step of drying the matrix-immobilized surfactant-coated lipase complex. Drying is preferably effected via freeze drying. Following freeze drying, the matrix-immobilized surfactant-coated lipase

According to a preferred embodiment, the matrix-immobilized surfactant-coated lipase complex represents 2-30 weight percent of the substrates. In another preferred embodiment the oil/fat substrates are liquid oils and solid fats. The oil may be any of the above listed oils in a native or hydrogenated form.

Further according to the present invention there is provided a triacylglycerol prepared according to the above process. The triacylglycerol serves an application such as a cocoa butter substitute, human milk fat-like, triglycerides for special diets or structured triglycerides for medical applications.

Yet, further according to the present invention there is provided a preparation which includes a lipase and an organic solvent. The lipase possessing both esterification (inter- and trans-esterification), acidolysis, alcoholysis and hydrolysis catalytic activities with respect to substrates, yielding esterification and hydrolysis products, respectively. The hydrolysis products represent less than about 7, preferably less than about 5, more preferably less than about 3 weight percent of the products.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

EXPERIMENTAL PROCEDURES

Materials:

Different crude lipase preparations were tested in this study. Table 1 below lists commercially available lipase preparations that were employed in this study, as well as their species source, protein content and supplier.

All fatty acids and triglycerides employed in this study were obtained from Fluka (Switzerland) and, as reported by the supplier, were at least 99 % pure.

Olive oil, sun flower oil, palm oil, canola oil, corn oil and *Nigella sativa* oil were obtained from local suppliers in the Galillee area, Israel.

Fish oil, tris(hydroxymethyl)aminomethane and the inorganic matrices used as supports for the surfactant-coated lipase complexes, including DE, alumina and silica gel were obtained from Sigma (USA).

Analytical grade *n*-hexane and other solvents employed, all of analytical grade, were from Bio Lab (Israel).

described in references No. 16 and 17, both are incorporated by reference as if fully set forth herein. A typical immobilized surfactant-coated preparation was prepared as follows. Crude lipase (300 mg protein) was dissolved in 1 liter tris buffer pH 5.5 containing 4 grams insoluble inorganic or organic matrix (DE, silica gel, alumina, or polypropylene). The enzyme solution was vigorously stirred with a magnetic stirrer at 10 °C for 30 minutes. Sorbitan mono-stearate (1 gram) dissolved in 20 ml ethanol was added dropwise to the stirred enzyme solution. The resulting colloidal enzyme solution was sonicated for 10 minutes and then stirred for 3 hours at 10 °C. The precipitate was collected by filtration or centrifugation at 12,000 rpm at 4 °C in a Sorvall Centrifuge, model RC-5B (Newtown, CT), followed by overnight freezing at -20 °C and then freeze-drying.

Lipase immobilization through ionic binding: Crude lipase (100 mg protein) was dissolved in 100 ml tris or phosphate buffer pH 5.5 containing 7 grams ionic exchange resin (Amberlite IRA-900 or IRA-95, Dowex or solfoxyethetylcellulose, all products of Sigma, USA). The enzyme solution was vigorously stirred with a magnetic stirrer at 10 °C for 30 minutes. Sorbitan mono-stearate (0.25 gram) dissolved in 2 ml ethanol was added dropwise to the stirred enzyme solution. The resulting colloidal enzyme solution was sonicated for 10 minutes and then stirred for 3 hours at 10 °C. The precipitate was collected by filtration or centrifugation at 12,000 rpm at 4 °C in a Sorvall Centrifuge, model RC-5B (Newtown, CT), followed by overnight freezing at -20 °C and then freeze-drying. All enzyme preparations prepared by this method contained 0.5-1.2 wt % protein.

Lipase immobilization through covalent immobilization: Two different immobilization procedures were adopted.

According to the first, the enzyme was primarily coated with a surfactant and then the lipase-surfactant complex was covalently linked to an Eupergit matrix which contains active oxirane groups.

To this end, crude lipase (1 gram protein) was dissolved in 1 liter tris or phosphate buffer pH 5.8. The enzyme solution was vigorously stirred with a magnetic stirrer at 10 °C for 30 minutes. Sorbitan mono-stearate (6.5 grams) dissolved in 30 ml ethanol were added dropwise to the stirred enzyme solution. The resulting colloidal enzyme solution was sonicated for 10 minutes and then stirred for 3 hours at 10 °C. Eupergit C or Eupergit C 250L (125 grams) and 12 ml solution of 5 % hydrogen peroxide were added into the enzyme solution and the resulting suspension was gently handshaken for a 1 minute, and then

Initial reaction rates were estimated from slopes of plots of the concentrations of LCTs at conversions less then 7 % as a function of time.

Unless otherwise indicated, all experiments were conducted under the above described conditions. Each esterification reaction was carried out in duplicate. In all experiments, *n*-hexane was dried over molecular sieves to minimize its water content down to 6 mg/liter. Thus, water concentration in all reaction systems, except for those where the effect of water was investigated, was less than 30 mg/liter.

Alcoholysis reactions: The alcoholysis reactions of long-chain triglycerides (LCT) with a long-chain fatty alcohol (LCFAL) to produce wax esters were initiated by adding 10 mg lipase preparation to 10 ml *n*-hexane that contained, typically, 25 mg LCT, such as tripalmitin and 50 mg LCFAL, such as cetyl alcohol. The reaction solution was treated similarly to the aforementioned acidolysis reaction.

Analytical methods: The concentrations of mono- di- and triglycerides were determined using a gas chromatograph (GC), HP 5890, equipped with a flame ionization detector (FID). A Capillary column, RTX-65TG, 0.53 mm ID x 30 m, 0.1 μm film thickness (RESTEK, Corp., PA, USA), was used under the following separation conditions. Injector and detector temperature were maintained at 350 °C, initial column temperature was 300 °C, followed by 1 minute isotherm, thereafter, the oven temperature was raised at a rate of 20 ° C/minute to 350 °C and this temperature was maintained for 5 minutes.

EXPERIMENTAL RESULTS

EXAMPLE 1

Effect of inorganic matrix on amount of recoverable lipase

Various sorbitan mono-stearate-lipases (surfactant-coated lipase) complexes were matrix-immobilized following the physical immobilization procedure described in the experimental procedures section above.

Matrix and lipase recovery efficiencies from the stirred enzyme solution for various combinations of lipases and inorganic matrices are reported in Table 2 below.

The lipases, Saiken 100 and lilipase gave the highest protein content in their respective surfactant-coated lipase complex as well as the highest amount of recovered precipitate. The other lipase preparations listed in Table 2 gave approximately the same protein content when only coated, about 7 %, and when both coated and immobilized, about 0.3 %.

TABLE 2 (Continued)

Lipase *Rhizopus Javanicus*

Inorganic matrix	Initial protein content [mg/l]	Inorganic matrix [g/l]	Sorbitan mono stearate conc. [mg/l]	Precipitate weight [gr]	Protein recovery [mg]	% protein recovery
without matrix	300	0	1000	0.2567	23.2	7.7
Alumina	300	4	1000	3.6	15	5
DE acid washed	300	4	1000	2.92	13	4.3
Silica	300	4	1000	2.567	12	4

EXAMPLE 2

Inter-esterification activity of inorganic matrix-immobilized surfactant-coated lipase complexes

Inter-esterification activity of crude lipase preparations was tested in *n*-hexane using tripalmitin (50 mg) as a triacylglycerol substance and capric acid (35 mg) as a medium-chain fatty acid substance. Inter-esterification activities of 10 mg non-immobilized surfactant-coated lipase (5-10 % protein content), or of 20 mg of inorganic matrix surfactant-coated lipase (0.2-2 % protein content), in *n*-hexane (10 ml), were examined using the same substrates.

The results indicate that in their native form the crude lipases used in this study lack measurable inter-esterification activity altogether under the low water conditions employed. In other words, in their native form the lipases acquire non-active conformation in the hydrophobic environment provided by the solvent (*n*-hexane).

When the crude lipases were modified to form surfactant-coated lipase complexes, as described in, for example, reference No. 3, all acquired inter-esterification activity with 1,3-positional specificity, under otherwise similar reaction conditions.

Figure 3 presents the conversion of tripalmitin with time when DE-immobilized surfactant-coated Saiken-100 (triangles) and lilipase A10-FG (squares) were used to catalyze the inter-esterification reaction of tripalmitin and capric acid. The inter-esterification reaction rates thus measured were 0.096 and 0.104 mmol/min·mg biocat., respectively.

All tested immobilized and coated lipases predominantly catalyzed the inter-esterification of tripalmitin and capric acid. The main products appeared in the reaction systems were 1-caproyl-2,3-dipalmitoyl glycerol (CPP) and 1,3-dicaproyl-2-palmitoyl glycerol (CPC). Under the described experimental conditions a steady state for both inter-esterification reactions was achieved within about 2 hours.

TABLE 3

Conversion of tripalmitin after 1h reaction time

Lipase Saiken 100	% conversion of tripalmitin after 1 h reaction
Crude Saiken 100	0
Surfactant-coated (modified) Saike 100	86
Modified Saiken 100 on alumina	29
Modified Saiken 100 on DE	68
Modified Saiken 100 on silica gel	60

Lilipase A10-FG	**% conversion of tripalmitin after 1 reaction**
Crude lilipase A10-FG	0
Surfactant-coated (modified) lilipase A10-FG	86
Modified lilipase on alumina	82
Modified lilipase on DE	81
Modified lilipase on silica gel	61

LipaseF AP-15	**% conversion of tripalmitin after 1 reaction**
Crude lipaseF AP-15	0
Surfactant-coated (modified) lipaseF AP-15	80
Modified lipaseF AP-15 on alumina	65
Modified lipaseF AP-15 on DE	75
Modified lipaseF AP-15 on silica gel	60

Lipolase 100T	**% conversion of tripalmitin after 1 reaction**
Crude lipolase 100T	0
Surfactant-coated (modified) lipolase 100T	60
Modified lipolase 100T on alumina	50
Modified lipolase 100T on DE	57
Modified lipolase 100T on silica gel	50

Lipase M	**% conversion of tripalmitin after 1 reaction**
Crude lipase M	0
Surfactant-coated (modified) lipase M	81
Modified lipase M on alumina	72
Modified lipase M on DE	75
Modified lipase M on silica gel	60

Lipase F-EC	**% conversion of tripalmitin after 1 reaction**
Crude lipase F-EC	0
Surfactant-coated (modified) lipase F-EC	80
Modified lipase F-EC on alumina	65
Modified lipase F-EC on DE	75
Modified lipase F-EC on silica gel	63

TABLE 4

Operational stability of non-immobilized and immobilized surfactant-coated lipase complexes (* R.R. = reaction rate)

Surfactant-Lilipase A10-FG	*R.R. 1st run (mmol/ min·mg biocat)	R.R. 2nd run (mmol/ min·mg biocat)	R.R. 3rd run (mmol/ min·mg biocat)	R.R. 4th run (mmol/ min·mg biocat)	R.R. 5th run (mmol/ min·mg biocat)
surfactant-lilipase	0.206	0.16	0.15	0.1	0.06
DE	0.104	0.10	0.095	0.095	0.094
Alumina	0.09	0.087	0.087	0.085	0.082
Silica gel	0.07	0.067	0.066	0.063	0.063

Surfactant-Saiken 100	R.R. 1st run (mmol/ min·mg biocat)	R.R. 2nd run (mmol/ min·mg biocat)	R.R. 3rd run (mmol/ min·mg biocat)	R.R. 4th run (mmol/ min·mg biocat)	R.R. 5th run (mmol/ min·mg biocat)
surfactant-Saiken	0.19	0.17	0.15	0.1	0.08
DE	0.09	0.085	0.086	0.084	0.084
Alumina	0.08	0.075	0.074	0.072	0.071
Silica gel	0.073	0.064	0.062	0.061	0.06

EXAMPLE 4

Fatty acid specificity of immobilized surfactant-coated lipase complexes

The specificity of the inorganic matrix-immobilized surfactant-coated lipase complexes of the present invention toward different fatty acid substrates was tested by monitoring the inter-esterification of fatty acids of various chain lengths with tripalmitin. The results are summarized in Table 5 below. Reaction conditions were essentially as described under Example 2 above.

From Table 5 it is learned that the immobilized surfactant-coated lilipase complexes according to the present invention predominantly catalyzed the inter-esterification of fatty acids and tripalmitin with 1,3-positional specificity. The concentration of hydrolysis products did not exceed 5 wt % of the initial tripalmitin concentration.

It is further learned that the inter-esterification activity of the inorganic matrix-immobilized surfactant-coated lipase complexes according to the present invention was affected by the fatty acid used as a substrate.

Thus, fatty acids having longer alkyl chains, such as palmitic and stearic acids, are better substrates for the DE-immobilized surfactant-coated lipase complexes than fatty acids having shorter alkyl chains. Similar data for non-immobilized lipases was obtained by others using different sources of lipases (19).

Table 7 below further demonstrates that the activity of DE-immobilized surfactant-coated lipase complex was affected by the HLB (Hydrophilic-Lipophilic Balance) of the surfactant bound to the biocatalytic complex.

Mixtures of sucrose-stearate esters (mono-, di- and tristereate sucrose esters) that have low HLB values gave biocatalytic complexes with low inter-esterification activity. On the other hand, when the HLB of the sucrose stearate esters mixture was selected high, an increase in the activity of the biocatalyst was observed. These results suggest that the mode of interaction between the enzyme and the sucrose fatty acid ester depends on the hydrophobicity of the sugar ester.

TABLE 7

The effect of the HLB of stearate sucrose esters of the complex on the activity the surfactant-coated lipase complex immobilized on DE

HLB	Inter-esterification rate (mmol/min·mg biocat
1	0.03
2	0.04
6	0.08
8	0.12
10	0.14
8	0.15
9.5	0.18
11	0.18
13	0.19
15	0.19
19	0.20

EXAMPLE 6

Temperature effect on the activity of inorganic matrix-immobilized surfactant-coated lipase complexes

The influence of the reaction temperature on the inter-esterification activity of the inorganic matrix-immobilized surfactant-coated lipase complexes according to the present invention was investigated in the range of 25-69 °C. Other than the temperatures employed, reaction conditions were essentially as described under Example 2 above.

As shown in Figure 4, the initial esterification reaction rates were determined and used in an Arrhenius plot.

Increasing the reaction temperature up to 50 °C led to increase in the reaction rate. Above 50 °C there was no significant increase, and at 69 °C there was very slight decrease in the reaction rate.

These results indicate that the inorganic matrix-immobilized surfactant-coated lipase complexes according to the present invention perform well also under elevated temperatures.

Steady states for both reaction systems were obtained within 4 h, wherein about 80 % of the tripalmitin was converted.

After 5 h, the reaction solutions were filtered to remove the biocatalyst and the *n*-hexane was evaporated.

The trans-esterified oil blends after evaporation of *n*-hexane were homogenous solids at room temperature and had a margarine texture.

Under the above experimental conditions less than 7 wt % of the initial total triglycerides were hydrolyzed to form the side products; mono- and di-glycerides.

These results are of potential industrial application. It is demonstrated here that margarines can be successfully produced by the proposed reaction model and therefore, substituting the hydrogenation process which leads to formation of unfavorable and unhealthy byproducts.

TABLE 9

Tripalmitin consumption during trans-esterification between liquid olive oil and solid tripalmitin

Time (h)	tripalmitin with surfactant-coated lipase Saiken100 on DE	tripalmitin with surfactant coated lilipase A-10FG on DE
0	100	100
0.15	74	72
0.5	61	50
1	46	36
2	32	25
3	28	21
4	23	17
5	19	16

EXAMPLE 9

Inter-esterification activity of surfactant-lipase complex covalently immobilized on Eupergit

Figure 5 shows the conversion of tripalmitin with time where the three different preparations of surfactant-lipase complexes of lilipase A10-FG and its crude form, were used in the inter-esterification reaction of tripalmitin and capric acid. All tested covalently immobilized lipases predominantly catalyzed the inter-esterification of tripalmitin and capric acid. The main products appeared in the reaction system were 1-caproyl-2,3-dipalmitoyl glycerol (CPP) and 1,3-dicaproyl-2-palmitoyl glycerol (CPC) were employed. The results show that the lipase which was first covalently attached to Eupergit and then coated with surfactant gave the highest inter-esterification activity. Also, the lipase which

TABLE 10

Conversion of tripalmitin after 2 hours reaction achieved by using various immobilized lipases.

Lilipase A10-FG

Enzyme form	Conversion (%)
Crude Lilipase A10-FG	2
Lilipase on Eupergit	4
Lilipase on Eupergit + SMS	19
Lilipase on Eupergit + SMS after 48h	26

Lipase LP

Enzyme form	Conversion (%)
Crude lipase LP	1
Lipase LP on Eupergit	2
Lipase LP on Eupergit + SMS	14
Lipase LP on Eupergit + SMS after 48h	18

Lipase PS

Enzyme form	Conversion (%)
Crude lipase PS	1
Lipase PS on Eupergit	2
Lipase PS on Eupergit + SMS	8
Lipase PS on Eupergit + SMS after 48h	12

It can be seen from Table 10 that different lipases show different inter-esterification activity when are treated similarly. This result is ascribed to the different sources of the lipases used. All crude lipases showed very low inter-esterification activity under the described conditions while their activity has slightly increased when they were covalently immobilized on Eupergit. It is interesting to notice that when lipases were coated with a surfactant their inter-esterification activity has significantly increased. The highest conversion of tripalmitin to its inter-esterification products with 1,3-positional specificity that was achieved after 2 h reaction, was when the lipases were first covalently immobilized on Eupergit and then coated with surfactant. Lilipase A10-FG coated with the surfactant and immobilized on Eupergit yielded the highest inter-esterification activity within the three lipases tested in this respect (Table 10).

EXAMPLE 10

Operational stability of the immobilized surfactant-lipase complexes

Table 11 below shows the inter-esterification activity in five consecutive runs with different crude lipases which served as controls, and the inter-esterification activity of the same lipases when covalently immobilized to an insoluble matrix.

Eupergit gives an active and stable enzyme preparation which can be readily recovered following the end of the reaction.

EXAMPLE 11

Inter-esterification activity of surfactant-lipase complex immobilized on ion exchange resins

Various ion exchange resins were tested as lipase immobilizers. Amberlite IRA-900, Dowex 22 and Ethylsolfoxycellulose gave the highest activity when lilipase A10-FG was used to catalyze the inter-esterification reaction of tripalmitin and capric acid in *n*-hexane.

Table 12 below shows the inter-esterification activity of lilipase A10-FG immobilized on various ionic exchange resins.

TABLE 12

Operational stability of surfactant-coated lilipase A10-FG immobilized on various ionic exchange resins in five consecutive runs using the same biocatalyst batch. Reaction conditions were as in Table 10

Lilipase A10-FG	Reaction rate 1st run (mmol/ h·mg·biocat)	Reaction rate 2nd run (mmol/ h·mg·biocat)	Reaction rate 3rd run (mmol/ h·mg·biocat)	Reaction rate 4th run (mmol/ h·mg·biocat)	Reaction rate 5th run (mmol/ h·mg·biocat)
Crude	0.002	0	0	0	0
Ambrelite-IRA-900 (strongly acidic)	7.2	7.0	6.9	6.8	6.8
Ambrelite-IRA-900 (strongly basic)	6.5	6.2	6.1	6.0	6.0
Dowex 22	7.3	7.0	6.9	6.9	6.9
Ethylsolfoxy cellulose	9.1	8.6	8.5	8.5	8.4

It can be seen that all lipase preparations with the different resins showed some activity losses following the first run and this is attributed to detachment of surfactant molecules from the vicinity of the surfactant-lipase complex. The activity after the second run was almost constant.

EXAMPLE 12

Use of immobilized surfactant-lipase complex in the resolution of optically active compounds

The use of lipases in the resolution of optically active compounds have been widely practiced to resolve or synthesize chiral products from racemic or prochiral compounds. The resolution of optically active compounds is based on hydrolysis or esterification/inter-esterification reactions aided with a lipase

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10. The lipase preparation of claim 9, wherein said fatty acid is selected from the group consisting of monolaurate, monomyristate, monopalmitate, monostearate, dilaurate, dimyristate, dipalmitate, distearate, trilaurate, trimyristate, tripalmitate and tristearate.

11. The lipase preparation of claim 9, wherein said hydrophilic moiety is selected from the group consisting of a sugar, a phosphate group and a carboxylic group.

12. The lipase preparation of claim 11, wherein said sugar is selected from the group consisting of sorbitol, sucrose, glucose and lactose.

13. The lipase preparation of claim 9, wherein said fatty acid and said hydrophilic moiety are conjugated via an ester bond.

14. The lipase preparation of claim 1, wherein said lipase is from a species selected from the group consisting of *Burkholderia sp., Candida antarctica B, Candida rogosa, Pseudomonas sp., Candida antractica A,* Porcine, *Humicola sp., Mucor miehei, Rhizopus javan, Pseudomonas fluor, Candida cylindrcae, Aspergillus niger, Rhizopus oryzae, Mucor javanicus, Rhizopus sp., Rhizopus japonicus and Candida antarctica.*

15. The lipase preparation of claim 1, wherein said lipase is from a microorganism.

16. The lipase preparation of claim 1, wherein said lipase is from a multicellular microorganism.

17. The lipase preparation of claim 1, wherein the preparation maintains lipase catalytic activity in an organic solvent.

18. The lipase preparation of claim 17, wherein said organic solvent is selected from the group consisting of *n*-hexane, toluene, iso-octane, *n*-octane, benzene, cyclohexane and di-iso-propylether.

19. The lipase preparation of claim 17, wherein said catalytic activity is selected from the group consisting of esterification, inter-esterification and

29. The method of claim 21, wherein contacting of said lipase, insoluble matrix and surfactant in said aqueous solution under step (a) is effected by:

(i) dissolving said surfactant in an organic solvent for obtaining a dissolved surfactant solution;

(ii) mixing said lipase and said dissolved surfactant solution in said aqueous solution; and

(iii) adding said insoluble matrix into said aqueous solution.

30. The method of claim 21, wherein said conditions for said formation of the matrix-immobilized surfactant-coated lipase complex include sonicating said aqueous solution.

31. The method of claim 21, wherein said insoluble matrix is selected from the group consisting of alumina, diatomaceous earth, celite, calcium carbonate, calcium sulfate, ion-exchange resin, silica gel, charcoal, Eupergit and ethylsolfoxycellulose.

32. The method of claim 21, wherein said surfactant includes a fatty acid conjugated to a hydrophilic moiety.

33. The method of claim 32, wherein said fatty acid is selected from the group consisting of monolaurate, monomyristate, monopalmitate, monostearate, dilaurate, dimyristate, dipalmitate, distearate, trilaurate, trimyristate, tripalmitate and tristearate.

34. The method of claim 32, wherein said hydrophilic moiety is selected from the group consisting of a sugar and a phosphate group and a carboxylic group.

35. The method of claim 34, wherein said sugar is selected from the group consisting of sorbitol, sucrose, glucose and lactose.

36. The method of claim 32, wherein said fatty acid and said hydrophilic moiety are conjugated via an ester bond.

46. The process of claim 44, wherein at least one of said substrates is selected from the group consisting of an oil, a fatty acid, a triacylglycerol and a fatty alcohol.

47. The process of claim 46, wherein said oil is selected from the group consisting of olive oil, fish oil, palm oil, cotton seeds oil, sunflower oil, *Nigella sativa* oil, canola oil and corn oil.

48. The process of claim 46, wherein said fatty acid is selected from the group consisting of medium and short-chain fatty acids and their ester derivatives.

49. The process of claim 46, wherein said fatty acid is selected from the group consisting of oleic acid, palmitic acid, linolic acid, linolenic acid, stearic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid and their ester derivatives.

50. The process of claim 44, wherein contacting said matrix-immobilized surfactant-coated lipase complex with said substrates is effected within a reaction reactor.

51. The process of claim 44, wherein said reaction reactor is selected from the group consisting of a tank reactor and a fixed-bed reactor.

52. A process of changing the physical properties of oils/fats by trans-esterification or inter-esterification between at least two oil/fat substrates comprising the step of contacting an insoluble matrix-immobilized surfactant-coated lipase complex with said substrates.

53. The process of claim 52, wherein contacting said matrix-immobilized surfactant-coated lipase complex with said substrates is effected in presence of an organic solvent.

54. The process of claim 52, wherein said matrix-immobilized surfactant-coated lipase complex represents 2-30 weight percent of said substrates.

ACIDOLYSIS REACTION

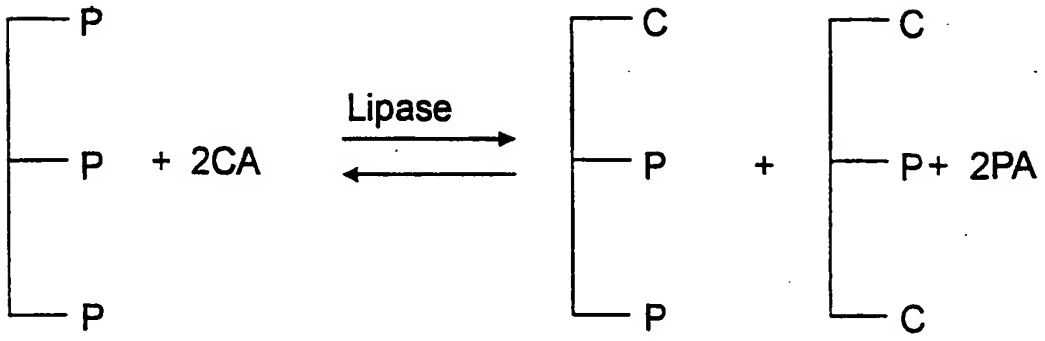

Fig. 1a

TRANSESTERIFICATION REACTION

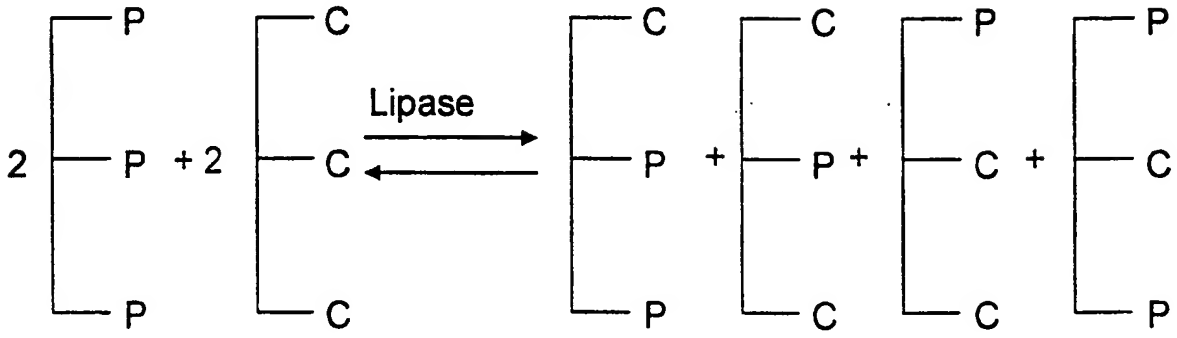

Fig. 1b

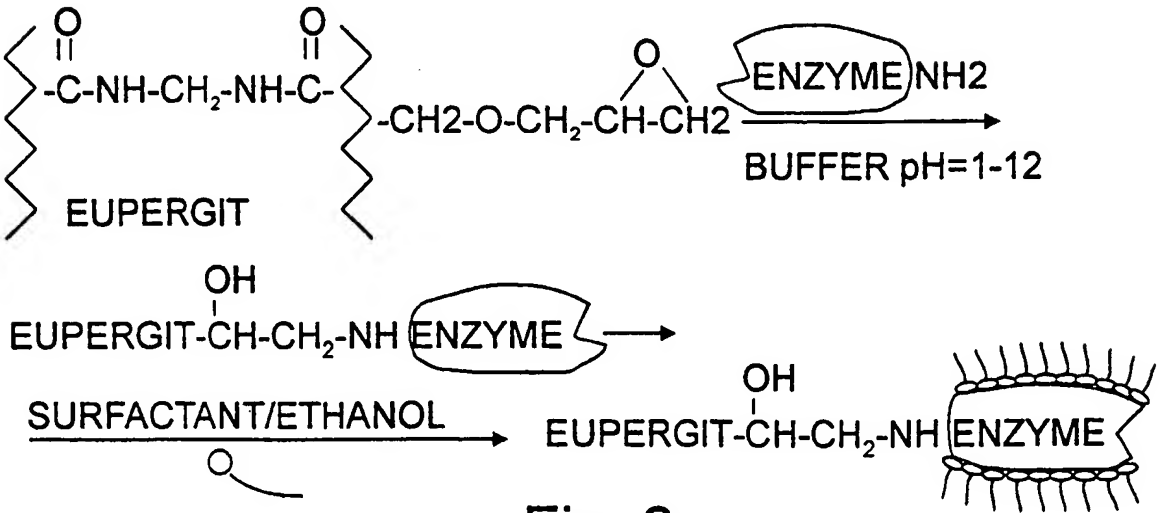

Fig. 2

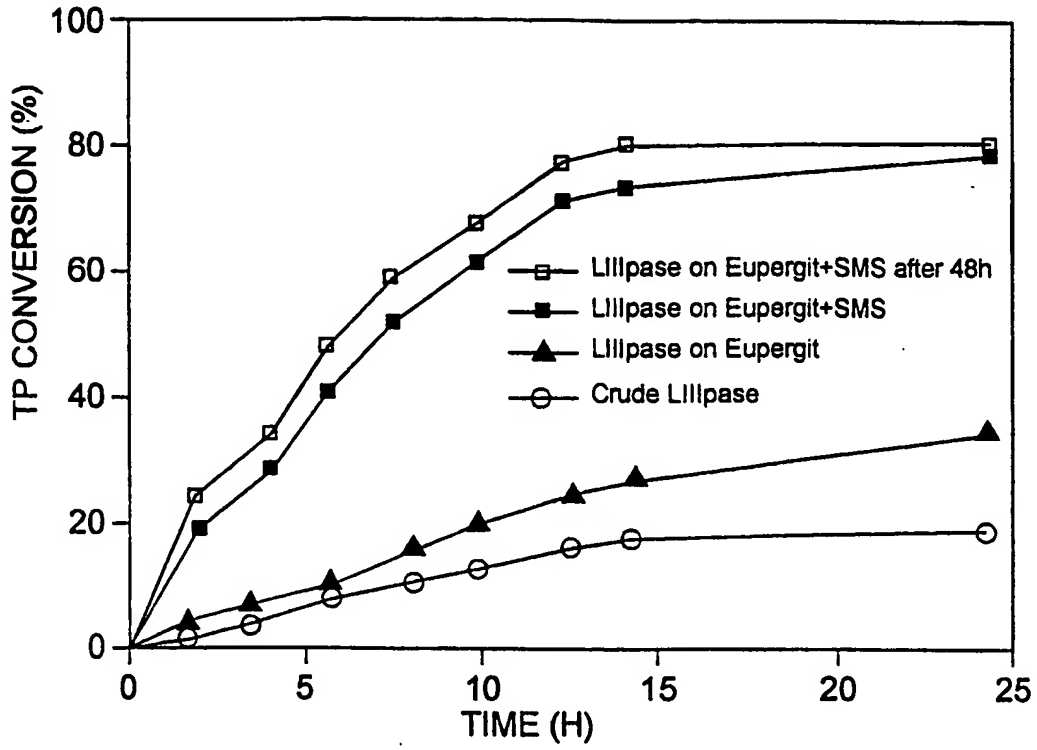
TP CONVERSION (%)
TIME (H)
LIIIpase on Eupergit+SMS after 48h
LIIIpase on Eupergit+SMS
LIIIpase on Eupergit
Crude LIIIpase

Fig. 5

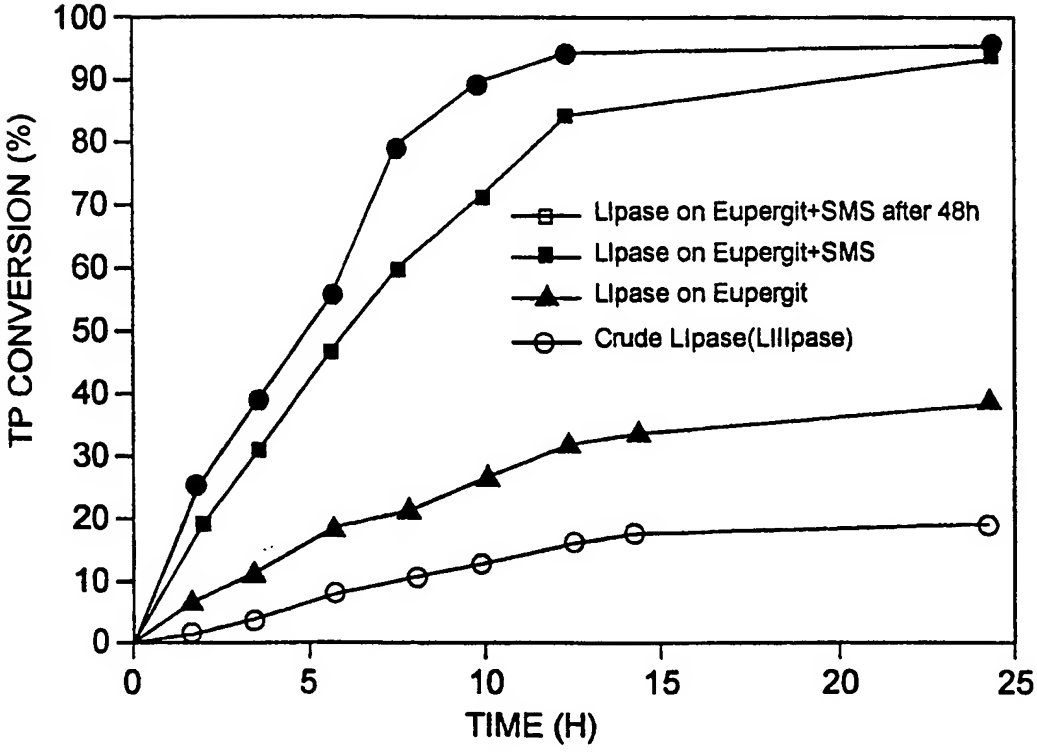
TP CONVERSION (%)
TIME (H)
LIpase on Eupergit+SMS after 48h
LIpase on Eupergit+SMS
LIpase on Eupergit
Crude LIpase(LIIIpase)

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15799

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	BASHEER et. al. Development of a Hollow-Fibre Membrane Reactor for the Interesterification of Triglycerides and Fatty Acids Using Modified Lipase. Process Biochemistry. 1995, Vol. 30, No. 6, pages 531-536, entire document.	58, 59 ---------- 1-57, 60

Claims of outstanding medical beneficial effects of the fish oils 7,10,13,16,19-docosahexaenoic acid (DHA) and 5,8,11,14,17-eicosapentaenoic acid (EPA), have been made in recent years (11, 17, 42, 47).

As a result, public demand for these ω-3 polyunsaturated fatty acids has increased. This, in turn, led to both commercial synthesis of these fatty acids and to the conduction of intense scientific investigations which focused on their pharmacological properties (10, 21, 31, 40, 42).

Although scientific research has confirmed that these fish oils are indeed beneficial to human health, unwanted side-effects such as vitamin A toxicity and obesity were also reported in the literature. Of particular interest are the reports by Reithmann *et al.* (41), and Nagy and colleagues (37).

Reithmann and his coworkers noted that DHA reduced the contraction velocity of cardiac myocytes induced by either stimulation of α1-adrenoceptors or β-adrenoceptors. Furthermore, these researchers showed that this acid decreases the availability of intracellular calcium in rat myocytes by depressing synthesis of the second messenger inositol-3-phosphate.

The study by Nagy and colleagues (37) reported DHA impaired basal glucose disposal and disrupted normal hormonal regulation of glucose uptake by rat muscle and adipose cells. Since glucose uptake into adipocytes is also linked to β3-adrenoceptors (15, 26, 48), collectively these reports suggest that fish oils could adversely affect adrenergic homeostasis as related to glucose metabolism and cardiovascular homeostasis.

If this is indeed the case, then there is a need to develop new structured triglycerides with both medium-chain and ω-3 polyunsaturated fatty acids that would be devoid of the adverse effects of the naturally occurring ω-3 polyunsaturated fatty acids, or saturated fatty acids.

Molecules of MCTs having one of their acyl groups substituted with an essential long-chain fatty acid would provide the nutritional advantages of both MCTs and LCTs.

For example, the acyl form of the polyunsaturated fatty acids, EPA, DHA or α-linolenic acid, incorporated on the *sn*-2 position of a certain triglyceride molecule with a medium-chain fatty acyl group on the *sn*-1 and *sn*-3 positions form a very useful triglyceride (38).

The aforementioned incorporated polyunsaturated fatty acids in the triglyceride molecules were shown to have several health benefits with respect to cardiovascular diseases, immune disorders and inflammation, allergies, diabetes, kidney diseases, depression, brain development and cancer.